2. MATERIALS AND METHODS

2.1 Media and culture conditions

Oatmeal agar

36.5 g oatmeal agar (Hi-Media, India) was added to 800 ml H_2O. The volume was made upto 1L, boiled and autoclaved.

YEG

Yeast extract	2 g
Glucose	10 g
H_2O	to 1 L

YEPD

Yeast extract	5 g
Peptone	10 g
Glucose	10 g
H_2O	to 1 L

Complete medium for Magnaporthe oryzae

Glucose	10 g
Peptone	5 g
Yeast extract	1 g
Cas amino acid (CAA)	1 g
$NaNO_3$	0.6 g
KCl	0.5g
$MgSO_4$	0.5 g

KH_2PO_4	1.5 g
H_2O	to 1 L
pH	6.5

Minimal medium for Magnaporthe oryzae

Glucose	10 g
$NaNO_3$	0.6 g
KCl	0.5g
$MgSO_4$	0.5 g
KH_2PO_4	1.5 g
H_2O	to 1 L
pH	6.5

AB minimal medium for Agrobacterium

AB liquid	90 ml
20 x AB buffer	5 ml (1 x)
20 x AB salts	5 ml (1 x)

AB buffer	(20 x)
K_2HPO_4 (anh.)	6 g
NaH_2PO_4 (anh.)	2 g
H_2O	to 100 ml

Each salt was dissolved separately in ~ 50 ml H_2O and then mixed to obtain 100 ml solution with pH 7.0.

AB salts (20 x)

NH_4Cl	2 g
$MgSO_4.7H2O$	0.6 g
KCl	0.3 g
$CaCl_2$ (anh.)	0.3 g
$FeSO_4$	0.005 g
H_2O	to 100 ml

AB liquid

0.5 g glucose in 90 ml H_2O

Induction medium for Agrobacterium

20 x AB salts	5 ml (1 x)
Glucose	180 mg (10mM)
Glycerol	0.5 % w/v
H_2O	to 100 ml
pH	5.3

Synthetic Dropout (SD) medium for yeast

YNB without amino acids (Hi-Media, India)	1x
Ethanol	2%
H_2O	to 1 l

Quadruple Dropout medium for yeast

YNB without amino acids (Hi-Media, India)	6.7 g
Glucose	20 g
Agar	20 g
Water	850 ml
10x Dropout Solution (His^-,Leu^-,Ade^-,Trp^-)	100 ml
Adjust pH 5.8	

10x Dropout Solutions (His^-, Leu^-, Ade^-, Trp^-)

Nutrient	**10x Concentration**
L-Arginine HCl	200 mg/L
L-Isoleucine	300 mg/L
L-Lysine HCl	300 mg/L
L-Methionine	200 mg/L
L-Phenylalanine	500 mg/L
L-Threonine	2000 mg/L
L-Tyrosine	300 mg/L
L-Uracil	200 mg/L
L-Valine	1500 mg/L

YE5S media

YEG	2.5 g
Dextrose	15 g

20x 5S Solution	50 mL
Water	950 mL

20x 5S Solution

Nutrient	**10x Concentration**
L-Histidine HCl monohydrate	200 mg/L
L-Adenine hemisulphate salt	200 mg/L
L-Uracil	200 mg/L
L-Lysine HCl	300 mg/L
L-Leucine	1000 mg/L

EMM media

Potassium hydrogen Phthalate ($KHC_8H_4O_4$)	3 g
Disodium hydrogen Phosphate (Na_2HPO_4)	2.2 g
Ammonium chloride (NH_4Cl)	5 g
D-glucose	20 g
Salt Stock (50x)	20 mL
Vitamin Stock (1000x)	1 mL
Mineral Stock (10,000x)	0.1 mL
Supplements (10x)	100 mL
Water	880 mL

Salt Stock (50x)

$MgCl_2$ .6 H_2O	53.5 g

$CaCl_2$. 2 H_2O	0.74 g
KCl	50 g
Na_2SO_4	2 g
Water	1 L

Vitamin Stock (1000x)

Sodium pantothenate	1 g
Nicotinic acid	10 g
Inositol	10 g
Biotin	10 mg
Water	1 L

Mineral Stock (10,000x)

H_3BO_3	5 g
$MnSO_4$	4 g
$ZnSO_4$. 7 H_2O	4 g
$FeCl_3$. 6 H_2O	2 g
H_2MoO_4. H_2O	0.4 g
KI	1 g
$CuSO_4$. 5 H_2O	0.4 g
Citric acid	10 g
Water	1 L

STC Solutions

D-Sorbitol	91 g
1M Tris-Cl (pH 8)	25 mL
1M $CaCl_2$	25 mL
Water	500 mL

PTC Solutions

Polyethylene glycol 3350	200 g
1M Tris-Cl (pH 8)	25 mL
1M $CaCl_2$	25 mL
Water	500 mL

CMS Solutions

Complete medium	1x
D-Sorbitol	1M

Top Agar

CAA	6 g
Yeast extract	6 g
Sucrose	200 g
Agarose	4 g
Water	1000 mL

Bottom Agar

CAA	6 g

Yeast extract	6 g
Sucrose	200 g
Agar	20 g
Water	1000 mL

2.2 Buffers

DNA extraction Buffers for M. oryzae

Tris-Cl (pH 8) 1M	15 mL
EDTA (pH 8) 0.5M	15 mL
NaCl (4M)	18.75 mL
After autoclaving add	
β- mercaptoethanol	105 µL

Lysis Buffer for E. coli

NaH_2PO_4 (100mM)	138 g
Tris (10mM)	1.2 g
Urea (8 M)	480.5 g
Water	1 L
Adjust pH 8 using NaOH	

Wash Buffer for E. coli

NaH_2PO_4 (100mM)	138 g
Tris (10mM)	1.2 g
Urea (8 M)	480.5 g

Water	1 L
Adjust pH to 6.3 using HCl	

Elution Buffer for E. coli

NaH_2PO_4 (100mM)	138 g
Tris (10mM)	1.2 g
Urea (8 M)	480.5 g
Water	1 L
Adjust pH to 5.9 using HCl	

Phosphorylation Reaction Buffer (1x)

Tris	50 mM
$MgCl_2$	10 mM
EDTA	0.1 mM
DTT	2 mM
pH	7.5

Dephosphorylation Reaction Buffer (1x)

NaCl	100 mM
Tris	50 mM
$MgCl_2$	10 mM
DTT	1 mM
pH	7.9

Phosphate Buffer Saline

NaCl	8 g
Na_2HPO_4	1.44 g
KCl	0.2 g
KH_2PO_4	0.24 g
Water	1 L
pH	7.4

IP Buffer

Tris	50 mM
NaCl	150 mM
Tween-20	0.05% (v/v)
Water	100 mL

PGSK buffer (1x)

$NaH_2PO_4. H_2O$	0.52 g
$Na_2HPO_4. 2H_2O$	8.8 g
Glucose	11 g
NaCl	2.83 g
KCl	0.372 g
Water	1 L

UTE Buffer (10mL)

Urea	4.2 g

Thiourea	1.5 g
Triton X 100	200 µL
DTT	200 mg
CHAPS	400 mg
PMSF (100mM)	100 µL
EDTA (500mM)	20 µL
Water	4 mL

Rehydration Solution (5mL)

Urea	2.4 g
CHAPS	0.2 g
Tris	0.024 g
DTE	0.05 g
Water	5 mL

2.3 Stock Solutions

Antibiotics

Ampicillin	100 mg/mL
Rifampicin	5 mg/mL
Tetracycline	10 mg/mL
Kanamycin	50 mg/mL
Hygromycin B	200 mg/mL
Cefotaxime	250 mg/mL

Reagents

PMSF	100 mM
EDTA	500 mM
Ammonium persulphate (APS)	10% (w/v)
IPTG	100 mM

2.4 Bacterial strains

Escherichia coli DH5α (F^-, *end*A1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *thi*-1, λ^-, *rec*A1, *gyr*A96, relA1 Δ(*lacZYA-arg*F) U169 *deo*R (ϕ80d*lac*Δ(lacz) M15; Bethesda Research Laboratories) was used for bacterial transformation and plasmid propagation. *Escherichia coli* BL21 (DE3) genotype F^- *omp*T *hsd*S_B(r_B^- m_B^-) *gal dcm* (DE3). The *E. coli* strains were grown at 37^{o}C on Luria-Bertani (LB) agar medium. *E. coli* transformants carrying plasmid vectors were grown on LB agar containing either Ampicillin or Kanamycin at a concentration of 100 μg/ml or 50 μg/ml, respectively, as required. Bacterial strains were maintained at 4^{o}C as slants or stab cultures on LB agar medium. Long-term preservation was under 25% glycerol solution at -70^{o}C.

2.5 Yeast strains

Schizosaccharomyces pombe strain CHP428/429 (*diploid skp1::ura4*$^+$) was used for the complementation study of *MoSkp1*. The yeast strain was grown at 28-30^{o}C and maintained on YE5S. YE5S was used as rich media and modified synthetic EMM2 as minimal media. Ampicillin was added to the autoclaved medium at a final concentration

of 200 µg/ml to avoid bacterial contamination. The genotype of yeast strain AH109 used in yeast two hybrid assay is MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : $GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2,URA3 : : $MEL1_{UAS}$-$MEL1_{TATA}$-lacZ.

2.6 Fungal cultures

Magnaporthe grisea B157 belonging to the international race IC9 was isolated in our laboratory from infected rice leaves (Kachroo *et al*. 1994). The fungus was grown on either YEG agar or oat meal agar, at 28-30^{o}C. Fungal conidia were harvested by scraping the biomass grown on oatmeal agar plates with a sterile surgical blade, resuspended in sterile water, and purified by passing through Miracloth (Calbiochem, USA). The different transformants were maintained as monoconidial isolates and stored as 25% spore glycerol stocks at -70^{o}C.

2.7 Competent cell preparation and transformation of *E. coli* with plasmid DNA

A single colony of *E. coli* DH5α was inoculated in 3 ml of Luria-Bertani broth and grown overnight. 100 ml of fresh LB broth was inoculated with 1 ml of overnight grown culture and grown at 37^{o}C till 0.3 to 0.4 $O.D_{600}$; chilled on ice and the cells were pelleted by centrifugation at 2012 g for 5 min at 4^{o}C. The cells were resuspended in ice cold 0.1 M $CaCl_2$ and incubated for 30 min. The cells were reharvested by centrifugation and resuspended in ice cold 0.1 M $CaCl_2$ with 20% glycerol. The culture was stored at -70^{o}C in 100 µl aliquots. For transformation, a 0.1 ml aliquot of this cell suspension was transferred to a 1.5 ml chilled microfuge tube; mixed with the plasmid DNA (usually 50

ng) or ligation mixture and incubated at 4^{o}C for 30 min. Cells were subjected to heat shock at 42^{o}C for 90 sec, followed by 5 min. incubation on ice. 1 ml of LB broth was added to the above suspension and incubated at 37^{o}C for 30 min. About 0.1 ml aliquot of transformation mixture was plated on LB agar medium containing either ampicillin (100 µg/ml) or kanamycin (50 µg/ml).

2.8 Transformation of *S. pombe* with plasmid DNA

The *S. pombe* strain Skp1 A7, was transformed with the plasmid pYES2-*MoSkp1* by 'One-step transformation' method (Chen *et al*. 1992). The culture was spread on a YEPD plate and incubated for 20-24 h. A small part of the growing culture was transferred using a sterile toothpick to a microfuge tube containing 100 µl of 45% PEG 3350 (solution in 0.1 M Lithium actetate, pH. 6.0 and 0.1 M DTT), 5 µl of 10 µg/ml of calf thymus DNA as carrier, which was sheared to give molecules in the 500 bp range. 1 µg of transforming DNA was added to each tube. The whole mixture was thoroughly vortexed and heat shock was given at 39^{o}C for 1 h. The cells were then gently spread on pre dried selection plates.

2.9 Protoplast transformation of *M. oryzae* B157

M. oryzae isolate B157 was inoculated in 50 ml YEG broth and grown for 3 day for each transformation experiment. The biomass was then filtered with Miracloth (Calbiochem, USA), washed with sterile distilled water and re-suspended in 50 ml sterile 1M sorbitol containing 50 mg lytic enzyme (Sigma, St. Louis, US). This was incubated overnight at 28°C at 100 rpm for protoplasting. Protoplasts were filtered, washed and resuspended in

200 µl of STC buffer (1M sorbitol, 50 mM Tris Chloride pH 7.4, 50 mM Calcium Chloride) containing 5 µg of plasmid DNA. After 1 h of incubation at 28°C, 1.2 ml of PTC (40% (w/v) Polyethylene Glycol 3550 (Sigma Aldrich, USA), 50 mM Tris Chloride pH 7.4, 50 mM Calcium chloride was added and incubated for another hour at 28°C under static condition before it was transferred to CMS (Complete medium with 1M Sorbitol) for 16 h at 28°C and 100 rpm. This suspension was mixed with top agar and plated on the selection plate containing 200 µg/ml Hygromycin B to select for transformants.

2.10. *Agrobacterium tumefaciens* mediated transformation of *M. oryzae* B 157

The *Agrobacterium* strain LBA4404 / pSB1 (Komari *et al*. 1996) was first transformed with respective vectors via triparental mating (Helper plasmid pRK2013; Helper strain *E. coli* DH5α). For triparental mating, single colonies, each from the freshly grown recipient *Agrobacterium* LBA4404 / pSB1, helper plasmid pRK2013 and the donor plasmid, were mixed together on YEP agar (Yeast extract 0.2 %, Peptone 0.5 %, agar 1.5 %) plates and incubated at 28^{o}C for 18-20 h. The mixture was scraped and re-suspended in 1 ml of 0.9% NaCl. 100 µl of serial dilutions of this suspension were plated onto selection plates and incubated at 28^{o}C for 2-3 days till the appearance of single colonies. The *Agrobacterium* harbouring T-DNA vector was then used to transform the fungus. *Agrobacterium tumefaciens* mediated transformation (ATMT) of *M. oryzae* was carried out as described (Mullins *et al*. 2001). Briefly, *M. oryzae* spores were collected from 7-8 days old oatmeal agar plates and spore concentration was adjusted to 1 x 10^{6} spores/ml. *Agrobacterium* carrying the *MoSkp1* antisense expression cassette was grown at 28^{o}C for

2 days in AB minimal medium supplemented with appropriate antibiotics. The *Agrobacterium tumefaciens* cells were diluted to OD_{600} = 0.15 in induction medium (IM) and grown for another 6 h, both in the presence (IM+AS) and absence (IM-AS) of 200 µM acetosyringone (AS). The cells were grown for an additional 6 h before mixing them with an equal volume of a conidial suspension from *M. oryzae* (1×10^5 conidia per ml). This mix (200 µl per plate) was plated on a 0.45-µm pore, 45-mm diameter nitrocellulose filter (Millipore, Bangalore, India) placed on co-cultivation medium (same as IM except that it contains 5 mM glucose instead of 10 mM glucose) in the presence of 200 µM AS. Following incubation at 28^{o}C for 2 days, the filter was transferred to YEG containing Hygromycin B (Sigma Chemical, St. Louis, MO, USA) at a final concentration of 200 µg/ml. as a selection agent for transformants and cefotaxime (250 µg/ml) to kill the *Agrobacterium tumefaciens* cells. Untransformed *M. oryzae* was kept as a control which did not grow on hygromycin containing medium. The transformants were maintained as monoconidial isolates to get pure cultures. Spores from these monoconidial cultures were stored in 25% glycerol at –70^{o}C until further analysis.

2.11 Isolation of plasmid DNA

Overnight grown culture (1.5 ml) was centrifuged in a microfuge tube and the bacterial pellet was resuspended in 200 µl of STET (8% w/v Sucrose, 5% v/v TritonX-100, 50 mM Tris-Cl pH 8.0, 50 mM EDTA pH 8.0). Lysozyme (10 µl of 50 mg/ml) was added to it, mixed by vortexing and boiled in a water bath for 45 sec followed by centrifugation at 13414g for 10 min. The resulting snot was removed with a tooth pick and 20 µl of 5% w/v CTAB was added followed by centrifugation for 5 min. To the pellet, 300 µl of 1.2

M NaCl was added and 750 µl of absolute ethanol was added. The DNA pellet was obtained by centrifugation for 10 min; washed with 70% (v/v) ethanol and resuspended in 50 µl of TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0).

2.12 Nucleic acid manipulations

Restriction enzyme digestion of DNA was carried out in 1x reaction buffers, supplied with the corresponding enzymes, as recommended by the suppliers. Blunt ended ligations were carried out at 20^{o}C, overnight, and sticky end fragments were ligated at 16^{o}C for 3-4 h using T4 DNA ligase.

2.13 Bioinformatics analysis of *MoSKP1*

Sequence sequestration and all the Bioinformatics analysis has been done using GROMO server (http://gromo.msubiotech.ac.in/Gromo/Complete_Search.cgi). Alternatively BLAST analysis was also performed in BROADMIT (http://www.broadinstitute.org/annotation/genome/magnaporthe_grisea/MultiHome.html) . The conserved domain prediction was done in NCBI and for phosphorylation site prediction NetPhos 2.0 program was used. PDB Map Viewer program was used for the 3D structure of the protein.

2.14 PCR amplification and cloning of *MoSKP1* gene

The phytopathogenic fungus *F. oxysporum Skp1* gene was used as a query sequence in the tBLASTx search tool of the National Center for Biotechnology Information (NCBI) to identify potential homologues in *M. oryzae*. A single hit was obtained from *M. oryzae* and other fungi. The highly conserved nucleotide sequence region was selected to design

the primers (*Hind*III RSkp1F 5´ TTG**AAGCTT**ATGTCAGAGGGTCAGCTGCA 3´) and (*Xho*I RSkp1R 5´ TTG**GAGCTC**ACGGTCCTCAGCCCACTC 3´) to amplify a 645 bp fragment of *MoSKP1* from *M. oryzae* B157 strain by PCR. This amplification product of *MoSKP1* was further used to make the RNAi construct. A blunt end ligation was done to generate KS-*MoSKP1* construct at the *Eco*RV site.

2.15 Generation of *MoSKP*1 RNAi, antisense, disruption and overexpression construct

In order to create RNA interference in *M. oryzae*, the pSilent Dual 2 vector (Nguyen *et al*. 2008), a derivative of the pSilent-1 vector containing two constitutive TrpC promoters in opposite orientations flanking the multiple cloning site, was used. This plasmid contained the hygromycin phosphotransferase gene (*hpt*) as a selectable marker. The gene fragment was amplified using XT-5 Polymerase. PCR was done to amplify a 645 bp by using specific primers containing restriction enzyme sites. The forward primer contained a *Hind*III site (F 5´TTG**AAGCTT**ATGTCAGAGGGTCAGCTGCA3´) and the reverse primer had a *Xho*I site (R 5´TTG**GAGCTC**ACGGTCCTCAGCCCACTC3´). PCR program used was as followed: Initial denaturation at 94ºC for 5 min, denaturation at 58ºC for 30 sec, elongation at 72ºC for 30 sec. This was repeated for 29 cycles and final elongation at 72ºC for 5 min. The PCR product was digested with *Hind*III and *Xho*I enzymes, gel purified and cloned at *Hind*III and *Xho*I site of pSD2 vector. Clones were confirmed by restriction digestion and PCR prior to fungal transformation. The plasmid construct was named pSD2-*MoSkp1*.

In an alternative approach, an antisense construct was made using the pSilent vector (Nakayashiki 2005, Nakayashiki and Nguyen 2008). The primer sequences were designed in such a way that the amplification product had *Kpn*I site at 5' and *Bam*HI site at 3' end. After amplification, the PCR product was digested with *Kpn*I and *Bam*HI and cloned at *Kpn*I and *Bgl*II sites (*Bam*HI and *Bgl*II have compatible ends) in the pSilent vector. Before fungal transformation, the construct was confirmed by restriction digestion and PCR. This construct was named pSilent-*MoSkp1*. For the generation of disruption construct the *MoSKP1* gene was first mobilised into a dual selection vector pGKO2 at *Kpn*I and *Spe*I site. The construct was then digested with the *Sal*I enzyme which cut in the middle of *MoSKP1* gene. A cohesive end ligation was done with the *hpt* fragment digested with the *Sal*I enzyme and the construct was named pGKO2-*MoSkp1* disruption vector. Over-expression construct was generated by cloning the PCR product in pSilent vector at *Kpn*I and *Hind*III site in sense orientation. Clone was confirmed by restriction digestion.

2.16 Triparental mating and mobilisation of antisense construct in *Agrobacterium*

The above antisense construct was used for fungal transformation by *Agrobacterium* mediated transformation. Triparental mating experiment was performed to mobilise the antisense construct into *Agrobacterium*. *Agrobacterium* LBA4404 strain used as recipient strain, an *E. coli* PRK2301 helper strain and DH5α containing antisense construct as donor strain. The three freshly grown cultures were used for the experiment. YEP agar (yeast extract 0.5% w/v, Peptone 1% w/v, Agar 2% w/v) media was prepared and a single colony from each culture was mixed together and kept for 36 h. After 36 h of incubation,

mixed culture was scraped and different dilutions were made. 100µl cells of $1X10^4$ dilution was spread on AB selection media containing Kanamycin (50 µg/ml), Tetracycline (5 µg/ml) and Rifampicin (10 µg/ml) as selection marker. Colonies appeared on the 3rd day and were inoculated in AB liquid media along with antibiotic with appropriate concentration and plasmid was isolated by alkaline lysis method. The isolated plasmid was again checked by restriction digestion to confirm the mobilisation of antisense construct into *Agrobacterium*. The *Agrobacterium* was also checked by Keto-lactose test to confirm the strain. *Agrobacterium* was streaked on YEL agar (Yeast extract 0.5% w/v, Lactose 1% w/v, Agar 2% w/v) and kept for two days at 28ºC. After growth of culture, freshly prepared Benedict solution ($CuSO_4$) was overlaid on it.

2.17 Cloning and heterologous expression of MoSkp1 protein in *E. coli* BL21DE3

The cloning of 6xHis-MoSKP1 fusion construct was achieved by the initial amplification of cDNA of *MoSKP1* and subsequent cloning into pBluescriptKS$^+$ vector (Invitrogen, CA, USA). PCR was performed from cDNA template using a proofreading enzyme XT-5 Polymerase (Bangalore Genei, Bangalore, India) and primers (Forward Primer: 5´ CGAT**GAATTC**ATGTCAGAGGGTCAGCTGCA 3´ and Reverse Primer: 5´ CGAT**GAGCTC**ACGGTCCTCAGCCCACTC 3´) that introduced *Eco*RI at the 5´ end and *Xho*I site at the 3´ end of the sequence. A 501-bp fragment (*MoSKP1* containing *Eco*RI and *Xho*I sites) derived from this vector was sub-cloned into pET30a vector (Novagen, Darmstadt, Germany). The clone was checked by restriction digestion and *E. coli* BL21DE3 was transformed with this construct. Positive clones growing on selection plate were selected for expression study. The culture was induced with 1 mM IPTG

(Isopropylthiogalactoside) and was kept for 4 h at 37ºC and 200 rpm; protein was isolated and checked for expression of MoSkp1 protein on 12% SDS PAGE stained with CBB R250.

2.18 Purification of *MoSkp1* and raising of polyclonal antibodies

Purification of MoSkp1 protein was achieved by using Ni-NTA affinity column (Qiagen, Darmstadt, Germany) following the manufacturer's instructions. The concentration of the recombinant protein was estimated by Bradford method (Bradford 1976). Polyclonal antibody was generated by immunizing a New Zealand White Rabbit using 100 µg purified protein and equal volume of Freund's Complete Adjuvant. Subsequently, two booster doses were given to the rabbit with Freund's incomplete Adjuvant and after 7 days the rabbit was bled and antiserum was collected and checked for the titer by ELISA and western blot.

2.19 PCR and southern blot analysis

After three successive selections of transformants on media containing Hygromycin B, presence of the transgene was confirmed by PCR. The PCR condition was optimised as initial denaturation at 94ºC for 5 min, denaturation at 94ºC for 30 sec, annealing at 62ºC for 30 sec, elongation at 72ºC for 1 min, 29 cycle repeat and final elongation was done at 72ºC for 5 min. *hpt* gene specific primers were used to screen the transformants (Hpt For - 5´AGGGCGAAGAATCTCGTGCTTTC3´) and (Hpt Rev- 5´CCACTATGGGCGAGTACTTCTAC3´). For Southern blot analysis, 10 µg of genomic DNA was digested with *Hind*III and 0.8% agarose gel electrophoresis was done to separate the digested products at 50 mV for 8 h. After denaturation and neutralisation

of the digest in the gel, capillary blot transfer was done for 16 h to transfer the DNA fragments onto a nylon membrane (Hybond N+, Amersham, US). The transferred DNA on the membrane was crosslinked by UV crosslinker (Spectrolinker, XL-1000, USA) at preset optimum cross linking program. A 350 bp fragment of TrpC promoter of the vector was amplified and the probe was prepared according to the manufacturer's instructions (Amersham, Buckinghamshire, US). The probe was hybridised with DNA on membrane for 16 h and after washing and substrate treatment an X-ray film was exposed for 4 h with the blot in Hypercassette (Amersham, USA). X-ray film was developed and documented.

2.20 Protein extraction, western blot hybridisation and MoSkp1 protein estimation

Protein extraction from various transformants was done by crushing the biomass of fungus into powder form and taking the extract in 1x PBS solution. PMSF was added to a final concentration of 1 mM to avoid proteolytic degradation of total protein extracted. Western blot analysis was done to detect relative levels of MoSkp1 protein in the RNAi (R1, R2, R3, R6, and R8) and antisense (A6 and A15.1) transformants. Total protein was extracted from wild type *M. oryzae* B157 strain and silenced transformants. The protein was estimated by Bradford method and 20 µg of total protein was used for Western blot. SDS-Polyacrylamide gel (12%) was run and transferred to PVDF membrane by wet transfer method. Ponceau S staining was done to check for equal loading of protein. Blocking was done with 3% skimmed milk and washed with Phosphate buffer saline pH 7.2, 0.1% Tween-20 (PBST). Rabbit polyclonal antibody (1:1000 dilution) against *Skp1* was used as primary antibody and HRP-conjugated Anti-IgG against rabbit was used as

secondary antibody (1:10000 dilution). The bands were visualised by providing substrate 3, 3' Diaminobenzidine (DAB) and hydrogen peroxide.

2.21 Total RNA isolation from transformants and quantitative analysis

Total RNA was isolated from the transformants and wild type strain B157 by crushing in liquid Nitrogen and taking into Trizol solution (Invitrogen, Thermo Fisher Scientific). Total RNA was purified and dissolved in DEPC treated Milli-Q water according to manufacturer's instructions. The quantitative PCR was performed on RNAi, antisense and over-expression transformants of *MoSKP1* in ABI 7900 HT Fast Real Time PCR System (Applied Biosystems, California, US) by monitoring increase in fluorescence of the SYBR Green dye in real time, according to the manufacturer's instructions. The primers designed (Skp1 real Forward 5´ GTTCTTGAGTGGTGTGA 3´), (Skp1 real Reverse 5´ GCATGAACTTCTGATCC 3´), (Mo β-Tubulin Forward 5´ GAGTCCAACATCAACGATCT 3´), (Mo β-Tubulin Reverse 5´ GTACTCCTCTTCCTCCTCGT 3´) for the amplification were selected from the C-terminal end of the mRNA, which amplifies 100 bp to ensure the detection of the remaining mRNA of *MoSKP1* transcript. The PCR program followed was: 10 cycles at 95ºC followed by 40 cycles, 10 second at 95ºC, 10 sec at 55ºC and 15 sec at 72ºC. Fold change of target gene transcript was calculated by using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{\text{gene of intrest}} - Ct_{\text{tubulin}})_{\text{test condition}} - (Ct_{\text{gene of interest}} - Ct_{\text{tubulin}})_{\text{control}}$, and relative abundance of target gene transcript was normalised to that of β-tubulin gene for each RNAi transformant as $2^{-\Delta Ct}$ were $\Delta Ct = (Ct_{\text{gene of intrest}} - Ct_{\text{tubulin}})$. Each RT-PCR quantification was carried out in triplicate and values for each gene were normalised to the expression level

of the respective control condition and used further to calculate the ratio of the expression level of the transcript.

2.22 Small RNA enrichment, polyacrylamide gel electrophoresis for RNA and detection of siRNA in *MoSKP1* transformants

For the detection of small RNA from transformants, total RNA was isolated using Trizol reagent (Invitrogen) and subjected to small RNA enrichment solution containing polyethylene glycol (PEG 8000, Sigma) at 50% (w/v) and sodium chloride up to 4 M concentration (Goto *et al*. 2003). Small RNA was precipitated overnight in absolute Ethanol. Small RNA was separated on 16% polyacrylamide gel for 2h at 100 mV and by electroblotting transferred to PVDF membrane (Amersham,USA). cDNA of *MoSKP1* was used as a probe prepared according to manufacturer's instruction (Amersham, Buckinghamshire, US).

2.23 Interspecific gene complementation study of *MoSKP1* in *S. pombe*

Interspecies gene complementation was carried out by expressing the *M. oryzae SKP1* in the s*kp1 A7* mutant of *S. pombe*. A vector was generated by sub-cloning cDNA of *MoSKP1* into yeast expression vector pYES2 under inducible GAL1 promoter for complementation in yeast. PCR was done to amplify a 501 bp DNA fragment using pBSKS-cDNA *MoSKP1* as template and proof reading enzyme XT 5 polymerase (Bangalore Genei, Bangalore, India). Primers used were as follows: mRNA Skp1 *Xho*I For 5´ CGAT**GAGCTC** ATGTCAGAGGGTCAGCTGC 3´ and mRNA Skp1 *Xba*I Rev 5´ CGAT**TCTAGA**TTAACGGTCCTCAGCCCA 3´. The PCR product was cloned into yeast expression vector pYES2 at *Xho*I and *Xba*I sites and yeast transformation was done

by Lithium acetate method (Daniel and Wood 2003). Putative transformants were selected on EMM medium containing 2% (w/v) galactose, without Uracil. The transformants were confirmed by PCR.

2.24 Cell wall and nuclear staining of *S. pombe* and rice blast fungus *M. oryzae*

All cells, wild type, mutant *skp1 A7* and complemented Skp1-pYES2-MoSkp1 were grown under induced condition (in presence of Galactose) at restrictive temperature of 37ºC. Cells were washed and fixed on glass slide and Calcofluor White (CFW) / Hoechst 33258 (Sigma) staining was done. The slides were observed under fluorescent microscope (BX51 Olympus) at 100x magnification. For visualization of nucleus and the cell membrane, Calcofluor white and Hoechst 33258 (Sigma) stain was used. Calcofluor white (Sigma) was dissolved in Milli Q water to make 1 mg/ml stock solution. Working solution made by diluting in PBS to final concentration of 10 µg/ml. After fixing, fungal mycelia and spores were incubated with PBS, 1% (v/v) Triton-X 100 solution for 2 h, washed with PBS and stain was applied for 10 min. Washing was done to remove remaining stain and slide was mounted in 50% glycerol. Hoechst 33258 (Sigma) stock solution was made by dissolving 1 mg in 1 ml Milli Q water. Working solution was made by diluting in PBS to final concentration to 2 µg/ml. Staining was done for 5 min at room temperature and slide was observed under microscope.

2.25 Immunolocalisation of MoSkp1 in *M. oryzae* B157

Spores were grown on hydrophobic slide and after appressoria formation, cells were fixed with fixing solution (formaldehyde 10% v/v, acetic acid 5% v/v, ethanol 85% v/v)

for ½ h followed by blocking solution (BSA 1% w/v) as described (Gupta and Chattoo 2007). Cells were incubated for 1 h with anti-MoSkp1 primary antibody (1:500 dilution); followed by washing and further incubated for an hour with Tetramethyl Rhodamine Isothiocynate (TRITC) conjugated secondary antibody (1:10000 dilution). After washing and mounting, the cells were observed under microscope Olympus BX51 at 100x magnification and picture were taken under 63x magnification of motorised confocal laser microscope (Carl Zeiss, LSM 700, Germany). Excitation of fluorescently labelled protein was done at 557nm and emission at 576 nm and images were captured using Charged-Coupled Device camera (AxioCam HR), under control of Zen software package. Image analysis was done with Zen software and Adobe Photoshop CS2.

2.26 Cell wall integrity assay

Cell wall integrity assay was performed to check the integrity of the cell wall in the *MoSKP1* RNAi transformants. Cell wall integrity of all the transformants was checked by growing on three cell wall disrupting agents: Caffeine, Congo Red and Calcofluor white (CFW) at concentrations of Caffeine (2.5 mM), Congo Red (2 mg/ml) and CFW (200 mM). The growth of fungus was monitored for 5 days and the growth area was measured as the diameter of the culture on plate and expressed as millimeter.

2.27 Infection assay, sporulation assay and appressorial assay of *MoSKP1* transformants

Plant pathogenicity test was performed by allowing spores to infect the rice leaves of CO39 rice cultivar. Spores from wild type *M. oryzae* B157 strain, OES1 and *MoSKP1* RNAi transformants were harvested and spore count was adjusted to $1x10^5$ spores/ml. 50

µl of spore were spotted on the adaxial surface of rice leaf and maintained at 24°C in 90% humidity. The development of lesions was observed 10 dpi and severity of infection was measured by counting lesion spots per unit area of leaf.

Onion epidermis assay was done to check the development of appressoria and further penetration of invasive hypha. Spore count was maintained at $1x10^4$ spores/ml and 50 µl was spread on single layer of onion epidermis and kept in dark for 12 h. Development of appressoria and progression of germ tube was observed under microscope and pictures were taken under 100x oil objective (Olympus BX51, Tokyo, Japan).

Appressorial development assay was performed by applying spores on a hydrophobic surface. Spore count was maintained at $1x10^4$ spores/ml and 20 µl of spore suspension was placed on cover slip (Esco, Erie Scientific) for 12 h. Development of appressoria was observed and pictures were taken under 100x oil objective (Olympus BX51) and 63x confocal LSM 700 (Carl Zeiss, Germany).

2.28 Hydroxyurea (HU) treatment of B157 strain and cell cycle study

Spores were isolated from B157 strain of *M. oryzae* and maintained at $1x10^4$ spores/ml. The spores were supplemented with 200 mM Hydroxyurea. Appressorial assay was kept for B157 *M. oryzae* without HU, B157 with HU, R1, R6, R9 and A15.1 transformant. Appressoria development was observed for 8 h and photographs were taken under microscope. Appressoria development of B157 without HU was taken as control.

2.29 Ubiquitinated protein enrichment assay and western blot analysis

Total protein of *MoSKP1* RNAi strain R6 and wild type B157 was isolated in 1x Phosphate Buffer. Protein was filtered through 0.22 µ membrane to remove cell debris. A 20 µg of total protein was loaded on protein enrichment column containing resin with affinity to ubiquitinated proteins. After washing, protein was eluted and mixed with sample loading dye. Sample was run on 12% SDS-PAGE and western transfer was done on PVDF membrane. Anti-ubiquitin antibody was used as primary antibody with 1: 1000 dilution. Secondary antibody (1:10000 dilution) used was anti-IgG conjugated with HRP. The detection was done using ECL method as instructed by manufacturer.

2.30 Phosphorylation and dephosphorylation assay of MoSkp1 protein

Purified 6XHis-MoSkp1 protein was used for phosphorylation and dephosphorylation assay. 120 µg purified 6XHis-MoSkp1 protein was taken and buffer was exchanged to 1x phosphorylation reaction buffer. In 400 µL reaction system, ATP was added to final concentration of 1 µg/µL and 5µL purified protein kinase A enzyme was added. The reaction mixture was kept at 16°C for 4h and the reaction was stopped by heating at 68°C for 15 min. The reaction mixture was divided into two vials, each with 200 µL reaction and buffer was exchanged to 1x dephosphorylation reaction buffer. Dephosphorylation reaction was carried out by adding Calf intestinal phosphatase (CIP) enzyme in one of the vials. Another reaction was kept without adding protein kinase A enzyme so as to see autophosphorylation. All the three reaction mixtures and one sample without any treatment were run on IEF and western blot analysis was done by transferring the protein on PVDF membrane. Bands were detected by using anti-Skp1 as primary antibody

(1:1000 dilution) which was further confirmed by using same blot with anti-His antibody (1:1000 dilution).

2.31 Pull down assay and yeast two hybrid assay

For pull down assay, MoSkp1-6xHis tagged purified protein was isolated in 1x IP buffer and total protein from wild type *M. oryzae* B157 was isolated in IP lysis (Immunoprecipitation) buffer and quantified by Bradford method. 120 µg purified MoSkp1-6xHis protein was mixed with 240 µg total protein of *M. oryzae* B157. The mixture was allowed to bind at 4°C with continuous rotation at 200 rpm overnight. After binding, the whole content was loaded onto 1.5 mL Ni-NTA resin. Three washings were done to remove loosely bound protein and then protein was eluted by using 400 mM Imidazole. The entire fraction was checked by SDS-PAGE to see the eluted protein fraction. Eluted proteins were precipitated by TCA precipitation and resuspended in UTE buffer. A pH 4 to pH 7 IPG strip was used for rehydration of the sample overnight. Isoelectric focusing followed by second dimension SDS-PAGE was performed using standard conditions. Gel was stained with Coomassie Brilliant Blue R 250. Purified MoSkp1-6xHis tagged protein was also run as control. The enriched proteins in pull down assay were selected and send for identification by peptide mass fingerprinting analysis.

Yeast two hybrid assay was performed by cloning bait (cDNA of *MoSKP1*, MGG_04978) and prey (cDNA of *MoFRP1*, MGG_06351) into pGBKT7 and pGADT7 vectors respectively. A co-transformation approach was used to transform *S. cerevisiae* cells (AH 109) using prey and bait vector. The transformants were able to grow on

quadruple dropout minimal media (SD: Trp^-,Leu^-,His^-,Ade^-) which is indicative of an interaction between the two proteins. The presence of both the proteins was confirmed by western blot analysis using anti-Myc and anti-HA as primary antibody.

2.32 Two dimensional polyacrylamide gel electrophoresis analysis of *MoSKP1* RNAi transformants

To compare the proteomes of *MoSKP1* RNAi R6 transformant and wild type B157 strain, a 2D gel electrophoresis analysis was done. Total protein was isolated from the R6 RNAi transformant and the wild type B157 strain by crushing the biomass in liquid nitrogen and isolating the protein in UTE buffer. About 100 µg total protein was rehydrated on non-linear IPG strip (pH 4 to 7) for 16h. Isoelectric focusing was done (Pharmacia Biotech, USA) according to manufacturer's instructions. Second dimension gel was run and CBB R250 staining was done to see the protein spots.